evolution

BOTANY REFERENCE NOTES

Paper – I: Morphogenesis

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Differentiation

1. Definition

Differentiation is a process at cellular level whereby a nascent unspecialised cell assumes a specialised structure and function in order to meet some specific structural or physiological requirement of the multicellular body. It is a significant process as it establishes specialised functions in different groups of cells in a multicellular body thereby bringing about division of labour which is the most important advantage of multicellular structure.

2. Characterization in Plants

The differentiation process among plants is characterized by –

- It is a location dependent process rather than lineage dependent as in animals.
- Largely governed by phytohormones which may act in the short or long range. There are no separate morphogenetic factors in plants unlike animals.
- Plants differentiation involves three types of cellular specialisation –
    a. **Specialisation by form transition-** For e.g., conversion of parenchyma into collenchyma.
    b. **Specialisation by partial cellular breakdown**– as in phloem sieve element cells in which nucleus, vacuole, and many protoplasmic components break down.
    c. **Specialisation by Programmed Cellular Death**– as found in case of xylary element formation and sclerenchymatous cells. This type of cellular specialisation is totally absent in case of animals.

3. Stability of Differentiation

Differentiation is essentially a stable process in plants requiring a change in gene expression pattern. However, two types of differentiation patterns have been identified on the basis of reversibility-

a. **Irreversible Differentiation or Plastic Differentiation –**

- Applies to xylary elements, sieve elements differentiation and sclerenchyma differentiation
- Irreversible because a mature sieve element lacks nucleus while a mature xylary element and sclerenchaymatous cell lacks proptoplasm altogether.

b. **Reversible or Elastic Differentiation –**

- Seen in all other examples of cellular differentiation except the above 3 and reproductive differentiation of meristem.
- Degree of reversibility is variable. It is the highest for early derivatives of mersitems, green parenchymatous cells and to an extent epidermal cells in younger parts just beneath the shoot apex. Other differentiated cells show a lesser degree of reversibility.

4. Stages of Differentiation

In all cases, it has 3 stages –

a. **Initiative or Inductive Stage**– where a cell is initiated for differentiation almost always by a phytohormonal signal.

5 phytohormonal signals are known to cause inducting stages –

- **Auxins** – promote vascular and fruit differentiation.
- **Cytokinin (CK)**– stimulates leaf ground tissue differentiation (mesophyll formation) and along with auxins CK may also stimulate vascular differentiation.
- **Gibberellins**– stimulate reproductive differentiation and elaboration of the embryo.

- **Ethylene**– stimulates vascular differentiation and differentiation involving PCD (Programmed Cell Death). E.g.– Aerenchyma, sclerenchyma formation.

- **Brasinisteroids**– required for mid or late stages of vascular differentiation.

b. **Execution Stages** – essentially the stages involving –

- **Biochemical Changes within the cell**– E.g. – Synthesis of new enzymes, cytoskeleton elements, new wall materials etc.

- **Physiological Changes**– E.g. – altered rates of photosynthesis, changed water and ionic relations, changed sensitivity towards light as morphogenetic factors, etc.

- **Structural changes**–E.g–Synthesis of secondary wall, wall thickening, change in cell shape, formation of or the obliteration of vacuole, etc.

- **Genetic changes in terms of differential gene expression**–E.g.– certain genes are stimulated while certain others are repressed such as STM, POL and WUS– genes interact to balance the rate of cell division (which enlarges the meristem) and the rate of cell differentiation in the periphery of the meristem (which decreases meristem size).

c. Outcome Stage–When a differentiated cell type is established, depending upon its protoplasmic and nuclear status, it may or may not differentiate.

While in a higher plant most of the differentiation involves gradual or subtle changes, the differentiation of xylem and phloem elements provide the most dramatic examples of major transitions

An example of differentiation: Xylogenesis

Xylem is the specialised tissue to conduct water and dissolved solutes from the roots to the aerial parts of the plants.

Xylem tissue has 3 elements –

a. **Conducting elements** – includes vessels and tracheary elements

b. **Parenchyma**– assists in radial translocation of water between xylem and phloem (which is essential for phloem functioning).

c. **Sclerenchymatous Fibres** – provide tensile strength to xylem tissue.

Of the above 3, the conducting elements are of highest physiological significance since they conduct water. For this task the conducting elements are microanatomically specialised.

Other features –

- **Absence of protoplasm** – hence no obstruction in conduction.
- **Thick walls with differential secondary thickening** – providing rigidity and preventing wall collapse even under high values of negative pressure arising due to transpirational pull.
- **Lignified walls**– because of which hydrophobic properties are rendered to the wall minimising the friction in water conduction.
- **Perforated region and pit formation**– facilitating conduction.

Xylogenesis is a process whereby a living derivative cell arising from procambial or cambial meristem goes through a differential process involving Programmed cell Death (PCD) to generate any one type of conducting element, i.e., tracheid or vessel. It occurs since seed germination till the late stages of the plant's life span. It is a location specific process affecting only the derivatives of procambium or cambium.

The Events in Xylogenesis – Xylogenesis is understood in detail based on the studies conducted in vitro in tracheary element differentiation of the species *Xenia elegens*. During in vitro studies, leaf mesophyll cells have induced to go through the steps of xylogenesis involving 3 stages –

a. Induction Stage –

- It initiates in immediate cambial derivative without any cell division.
- Inducing factor – relatively high auxin concentration with respect to CK concentration. This situation arises when after cambial meristematic activity the CK levels begin to fall while auxin continues at the same level.

- Auxin now stimulates ethylene synthesis. Ethylene, after synthesis, escapes the cells and then acts through its own receptors. It stimulates the induction related genes. The genes activated in this stage include –
    - ZeRT
    - $ZePTI_1$
    - $ZePTI_2$

Above 3 – earliest genes to express after ethylene signal.

The product of these genes stimulate the genes related to ribosomal protein, elongation factor proteins and tubulin proteins.

Protein synthesis commences in the cambial derivative cells and it behaves almost like a dedifferentiated cell.

Tubulin synthesis leads to microtubule polymerization which deposits on the inner face of primary wall and guides the deposition of the secondary wall.

The late genes to be activated in the process includes–TED 2, TED 3, and TED 4 – arrest potential of the cell to divide or grow further.

After this the lysis of the vacuolar membrane occurs.

b. Execution Stage or Re-Differentiation Phase –

In this stage, following groups of protein are active –

- Proteins involved in secondary wall maturation.
- Proteins involved in wall lignification.
- Proteins related to PCD, especially, cysteine proteases, DNAses and RNAses.
- Proteins involved in enhancing enzyme activity. E.g. – Calmodulin, which works with assistance of Ca^{++}.

Experimental studies reveal that the most active hormone in execution phase is a set of Brassinosteroids.

c. Outcome Stage –

This phase is the result of enzymatic and protein action in the execution phase. Following changes occur here –

- Thick secondary wall with lignin deposition.
- Disappearance of all the cytoplasmic compartments including nucleus.
- Breakdown of cytoplasmic materials like protein, lipid deposits, etc.
- Cellular elongation
- A continuity with the cell above and cell below in case of xylary vessels due to selective breakdown of upper and lower wall material.

The linearity of xylem differentiation is maintained by a polar pattern of auxin transport which occurs from cell to cell using specialed auxin efflux proteins called PIN proteins.

The important difference between vessel and tracheary element is the presence of perforation plate in vessels.

Polarity

1. Definition

Polarity is a definite developmental attribute of most of the organisms. It essentially means having two opposing points in the organisms body which follow different developmental designs. The straight line that connects any two opposing poles is called the axis.

2. Occurrence of Polarity

The anterior-posterior or apical-basal polarity is nearly universal in all the plants or algal groups with a few algal exceptions only. For e.g., Chlorella, Spirogira etc.

Dorso-Ventral polarity is relatively limited to lower bryophytic taxa and tracheophyte leaves.

3. Types

The living organisms can show 3 pairs of poles and consequently three types of polarity and axis –

a. **Anterior-Posterior or Apical-Basal Polarity** – With reference to plants it is also called shoot-root polarity also. If the plant concentration is prostrate then the term anterior-posterior polarity is applied.For eg. – In case of Hepaticophyta and Anthocerotophyta members. When the plant body is upright in growth as seen in mosses and all the tracheophytes, the term apical-basal polarity is applied.

b. **Dorso-Ventral Polarity** – is the differentiation of the plant body or any of its structure along two opposing plains – the dorsal and ventral plains. Tracheophyte plant bodies never show dorso-ventral polarity but the members of hepaticophyta and anthocerotophyta always have a dorso-ventral body. Among the tracheophytes, foliage leaves are mostly dorso-ventral in morphology (although anatomically, they can be isobilateral or dorso-ventral).

c. **Left-Right Polarity** – also called lateral polarity. Not observed in any plant group.

4. Establishment of Polarity

a. **Apical-Basal Polarity Establishment** – It is the best studied type of polarity establishment and it occurs very early during development. Eg. – In filamentous or anchored algae, the first division in a zygote or a settling zoospore is a longer transverse plain and it is asymmetric. The upper cell receives nucleus, most of the cell organelles and plastids and it gives rise to rest of the filamentous structures. The lower cell receives nucleus, a few cell organelles but not the plastids and it is always concerned with the development of anchoring or absorptive structures like rhizoids or heptera. Genera showing this behaviour – Ulothrix, Oedogonium, Chara, Fucus, Laminaria, etc. Fucus in this regard has been a model genus in scientific study.

In angiosperms, apical-basal polarity is also established with the first asymmetric division in the zygotic cell which is well characterized in most of the species. The apical and basal cells produced by zygotic divisions have distinctly differentiated developmental fates where most of the embryo results from the apical cell derivatives and the contribution of the basal cell is predominantly in the formation of the suspensor.

b. **Dorso-Ventral (DV) Polarity Establishment** – Best studied in the leaf of tracheophytes. The leaf can be anatomically isobilateral or DV but morphologically it mostly has a DV polarity.

The botanists use morphological terms adaxial (synonymous to morphological ventral side) and abaxial (morphological dorsal side) in the description of leaves. This polarity is established in the leaf during the mid-stages of leaf development.

5. Cellular, Physiological and Genetic Basis of Polarity

Cellular Basis – Polarity is established by 3 differential events at the cellular level –

a. **Different directions of Growth and division** – It is seen best in case of Apical-basal polarity where the SAM and the Root Apical Meristem (RAM) divide opposite to each other which increases the distances between the opposing poles.

b. **Different rates of Cell Division** – Also well established in apical-basal polarity where the apical meristematic derivative divides faster than the basal or root meristematic derivative.

c. **Different pattern of Specialisation** – Well studied in case of both apical-basal and DV polarity. The apical derivates are differentiated for assimilation or reproduction whereas the basal derivatives are specialised for the anchorage and absorption functions.

In leaf DV polarity, the pigment distribution, stomatal differentiation and epidermal specialisation, vary between the dorsal and ventral poles.

Physiological Basis– Physiologically, apical-basal polarity is better characterised. It includes the phytohormones auxin and cytokinin. The major site of action for both the hormones is the apical part. Hence, the apical part grows faster. Due to the presence of auxin efflux proteins called PIN, the movement of auxin is always from morphological apex towards morphological base thus creating a gradient of auxin distribution. This gradient specifies the root and the shoot poles because the region with low auxin presence differentiates into the basal meristematic region.

Genetic Basis – Genes have been identified for both the types of polarity determination in plants.

In apical-basal polarity determination, the maternally encoded proteins play the initiating role. For eg., SIN 1 gene in *Arabidopsis*. The SIN protein activates genes like SUS-1, SUS-2 in *Arabidopsis* and RASPBERRY gene in *Zea mays*.

Other genes involved include –

- GNOM – maintains axial polarity;
- MONOPTEROS – helps in root apical meristem formation;
- HOBBIT – regulates root apical meristem activity.
- SHOOTMERISTEMLESS – regulates SAM activity;
- TOPLESS – coordinates the RAM and SAM differential rates of division.

The genes involved in DV leaf polarity have recently been identified by McConell (2001), Byrne (2005). In *Arabidopsis*, 2 genes– (a) PHABOLUTA & PHABOLOSA or PHB1 or PHB2 are active in the adaxial side, whereas (b) the genes KANADI and YABBI are active in the abaxial side.

Both the gene products tend to repress each other, i.e., the region where YABBY & KANADI are abundantly active, the PHB1 & PHB2 cant show action and vice versa. Thus, during development, morphological DV polarity is well maintained.

6. Significance of Polarity

Polarity is one of the most important developmental determinants in plants where it ensures a proper positioning of the plant organs. For eg., the leaves and the reproductive structure always develop from the apical derivatives which is essential for biochemical assimilation and reproduction. Similarly, entire root system develops from the basal polar system which is also the appropriate location from the consideration of anchorage and absorption.

Symmetry

1. Definition

The term refers to a body plan along an imaginary plain on both sides of which the organisms body, if sectioned, would give rise to equal or highly comparable halves. Symmetrical establishment is an essential developmental aspect in most but not all the groups of organisms.

2. Types of Symmetrical Planning

a. **Asymmetric Body Plan**– i.e., the lack of any symmetrical plain that divides the body into two equal halves.

b. **Radial Body Plan**– In which an elongated or a spherical body can be cut along any vertical longitudinal plain/section (VLS) and generates two equal or highly comparable halves.

c. **Bilateral Body plan**– In which there is a single permissible plain of symmetry along which only a section can be taken and two equal or comparable halves would be generated after V.L.S.

3. Occurrence of Symmetry in Plants

Morphologically, all the 3 kinds of symmetrical arrangements can be observed among the plants. Yet, asymmetrical plan at maturity is most prevalent in higher plant divisions due to indeterminate branching & leaf emergences.

a. **Asymmetrical Plan**– Found in most of the Pteridophytes and Angiosperms because of somewhat uncertain number of branches on either side of the symmetric plain.

b. **Radial Plan** – is most closely achieved by normally growing conifers, cycads, the gymnospermous genus Welwitschia and certain monocot angiosperms especially those with repressed branching like the palms.

c. **Bilateral Plan** – is rare but present in the liverworts– both thallose and leafy.

Despite a mixed pattern of morphological symmetry, histologically, all the tracheophytes and the mosses show a radial body plan which is apparent by display of concentric zones of tissues seen in a transverse section through the stem and roots. Only certain anomalies disturb this radial histological planning. For e.g.–the formation of wedged-shaped secondary tissue in Gnetum sps. Disturbs the radial plan and converts it into bilateral structure.

4. Establishment of Symmetry in Higher Plants

Radial symmetry establishment requires concentric developmental fields to arise from a cylindrical mersitematic axis during embryogeny. This plan of development is conserved in all the vascular plants but best studied in angiosperms.

5. Cellular Basis

According to recent evidences, a concentric pattern of plant body development is laid down during the globular embryo stage. The 3 concentric regions are–the dermal, the ground and the cambial protomeristems. Till the level of globular embryo, the cells remain somewhat interconnected yet due to elimination of plasmodesmatal connections gradually there is restricted transport of materials among the cells.

Cellular analysis shows that the cells of one region remain connected to each other through plasmodesmata but similar connections are not available across the developmental regions.

This pattern of cellular interconnection leads to same types of activities by the cells belonging to a layer. The synchronous behaviour of cells within the globular embryo further enforces the concentric development plan of the embryo at the heart shaped stage. After the heart-shaped stage, the meristematic elaboration follows the same plan leading to a radial histology.

Physiological Basis– The hormonal control of radial patterning is not well characterized. Yet, 3 phytohormones have well described roles –

a. **Auxin**

- Stimulates apical meristematic activity.
- Increases sensitivity towards cytokinesis.
- Replaces excessive lateral enlargement of axial meristem

b. **Cytokinins**

- Stimulates cell division in presence of auxins
- Stimulates lateral expansion by increasing the rate of cell division in the ground meristem and protodermal regions.

c. **Gibberillic Acid**

- Responsible for tier-wise growth in the axial meristematic region

The hormones not only play a role by being present or absent but they also govern activities by their gradients. Auxin has a well characterized mode of gradient based action.

Genetic Basis– Many genes are not identified to be directly associated with symmetry, yet genetic genetic analysis of ***Arabidopsis thaliana*** shows the following genes-

a. **Keule** – responsible for normal development of protoderm.

b. **Pid (Pinoid Protein Kinase)**–which control the distribution of PIN proteins in a differential manner. This is essential for maintaining auxin gradient.

c. **STM (shootmeristemless)** – which identifies the apical meristematic precursors and enables them to proliferate into apical meristematic cluster.

6. Significance

a. Symmetry formation ensures correct positioning of the histological zones and thereby ensures physiological coordination among different tissues.

b. Whenever the plant body branches laterally, it follows the same symmetric plan as of the main axis. Thus, a pre-established symmetry establishes a developmental constraint over growth so that even in newly developed structures there is always a physiological coordination.

Totipotency

1. Meaning

Ability of a living isolated cell from a multicellular organism to initiate a developmental process (involving ordered cell division and temporally and spatially controlled differentiation) which eventually results into the regeneration of an entire munticellular individual of the same specie is called Totipotency.

The phenomenon is well established and widely spread in the plant world, especially the Bryophytes and the Angiosperms.

2. Discovery and Early Characterization

Discovered and physiologically characterized in angiospermic plants only with the landmark experiment of Gottlieb Haberlandt in 1902 in which he regenerated an entire plant of ***Daucus carota*** using a single, isolated meristematic cell as the starting material.

Early characterization– Following the experimentation of Haberlandt, scientists across the world replicated the experiment by varying the following 3-

- Source plant
- Source tissue
- Culture conditions (this parameter could be manipulated the least because plant cells usually have a uniform growth requirement)

The results from several experiments established the following facts –

1. Totipotency is found not only in all the groups of plant world but also in green, brown & red algae.
2. Totipotency is strongly established in two plant groups, viz., Bryophytes and angiosperms and weakly in Pteridophytes and Gymnosperms.
3. Totipotency always demands a carefully controlled growth environment. In the wild, isolated or detached single cells never regenerate into the entire plant.
4. Among the angiospermic families, most strongly totipotent plants are found in Solenaceae and Brassicaceae and least totipotent cells are confined to Myrtaceae.

3. Salient Features

1. In the entire range of biological diversity, plants show maximum degree of totipotency. In any animal phylum, an isolated single cell from a mature body can't be stimulated to regenerate the entire individual which living and nucleated plant cells do so frequently.
2. Totipotent regeneration is not totally autonomous but essentially it requires a balanced nutrient medium like MS medium (1978). And the presence of some essential phytohormones for this purpose which are auxin and cytokinin.
3. The Cellular requirements for totipotency is 3 fold –
   - Cell must be alive
   - Cell must be nucleated

- Cell must be having differentiated plastids or pro-plastids.

4. Totipotency is not equal to its degree but varies from the cell type to cell type and also according to the source plant species. The general rules are –

- Meristematic cells, especially SAM (Shoot Apical Meristem) or Axillary meristems have the highest degree of totipotency.
- Other green aerial tissues also have totipotent cells but less in regenerative abilities than SAM.
- The herbaceous plants have a greater abundance of totipotent cells especially herbaceous dicots. The best example is tobacco where nearly all cells except–phloem sieve tube elements, xylary elements, sclerenchymatous elements, root cap cells, root epidermal cells and the stomatal guard cells - show totipotent abilities.

4. Types of Totipotency

Totipotency has not been typified in plants as systematically as that done for animals. Yet, based on a review of this phenomenon in many plant species, Van and Tinh, in 1990, identified two types of totipotency shown by plants –

1. **Absolute Totipotency –**
   - Isolated cell can regenerate the entire plant body under appropriate condition.
   - Most frequent type found in plants.
2. **Semi Potency –**
   - A cell would give rise to only the plant organ which has provided the totipotent cell.
   - Thus, entire plant not regenerated.

Absolute totipotency differs significantly in its degree as explained by the ***Apical to Basal Totipotency Gradient Model*** of Wang and Phillips (1984). According to this model, the totipotent abilities progressively diminish when one moves from SAM towards the root tissue. The decline is

both in terms of totipotent nature (i.e., from absolute totipotency towards semipotent behaviour) and the effiency of totipotency, i.e., about 60% at SAM to about 2% at root apex.

5. Cellular, Physiological and Genetic Basis

Cellular Basis– Cells showing totipotency invariably shows three attributes– being alive, being nucleated and having plastids or proplastids.

Apart from the above 3 essential features, other attributes include –

a. Being relatively undifferentiated because with increasing level of differentiation totipotent abilities keep diminishing.

b. Having relatively undifferentiated wall.

c. Having a small size.

d. Having a small vacuole.

A violation of any one or more of the above criteria compromises totipotent ability.

Physiological Basis – Totipotency is governed by 3 types of physiological factors –

a. **Phytohormonal Factors** – (i) Auxins and Cytokinins favour totipotency. (ii) ABA represses it. (iii) Under stress, ethylene can also stimulate totipotency.

b. **Environmental Factors** – (i) Abundant nutrient supply, (ii) gradual loss of water within tolerable limits, (iii) gradual creation of hypoxic environment – all stimulate totipotent behaviour.

c. **Pathogenic Factors**– especially certain types of transforming bacterial pathogens like (i) ***Agrobacterium*** sps and (ii) infection by ***Cornybacterium*** (causes Witches Broom). (iii) ***Rhizobium*** infection prior to legume nodulation in the root is non-transforming in nature but it stimulates semi-potent behaviour in root cortical cells.

Genetic Basis– Cells continue to retain their zygotic genetic material generation after generation. Yet, at different stages and at different locations, they have different gene expressions. A differentiated cell normally loses expression of its genes related to cell division. If these genes are reactivated and

simultaneously, the genes related to differentiation function are silenced, the cell can become totipotent again from its differentiated form.

Operation of Totipotency

Differentiated Cell (Starting Material)

↓

Loss of Differentiation (De-Differentiation)

↓

Activation of Proliferation related genes

↓

A reverted of de-differentiated cell

↓

mitogenic stimulus (such as auxin/cytokinin/ethylene)

↓

Division

↓

Series of Division (hormonal control)

↓

Differentiation (Controlled by hormones)

Significance

a. Totipotency is the basis of certain types of responses from plants, especially vegetative propagation, wound healing, root proliferation under stress condition, etc. In natural conditions, totipotency is largely concerned with semi-potent activities.

b. Absolute totipotency is a powerful principle on which the discipline of plant biotechnology largely stays. At a late stage of most of the plant transformation experiments, there is a need to regenerate the entire transgenic plant from a small group of transgenic cells which would be impossible without totipotency.

c. At the level of scientific understanding, totipotency establishes that essentially all the living cells of the plant body have the same genetic content (principle of genomic quivalence). It is for this reason that even certain differentiated cells can be stimulated to regenerate the entire plant.

Cell Culture

What is plant cell culture?

1. The technique of maintaining and growing isolated plant cells on artificial medium under suitable conditions in order to:
    a. Investigate the properties and potentialities of plant cells
    b. Isolate some natural chemical product of interest such as secondary metabolites etc.
    c. Bring about biotransformation; and
    d. Select the desirable mutants or cell lines in crop improvement programmes
2. Care to be taken that the cells under culture do not form callus – hence the cultures are to be agitated at 100-250 rpm at regular intervals
3. The growing cells are suspended in the liquid culture medium – hence also called **suspension culture**

The explant

1. **Leaf tissue**: Most suitable, due to largely homogeneous population of cells which offer good conditions for raising defined and controlled cultures.
2. **From cultured tissues**: Most widely applied mode; uses calluses generated by isolated and cut plant organs.

Types of cell cultures

There are 2 main types.

Batch cultures

- Cells regularly transferred into new culture vessels containing fresh media (after the cells in the culture have touched the early *stationary phase*). Thus practices **sub-culturing**
- Important because cells begin to die in due course of time due to exhaustion of essential growth factors or accumulations of toxins.
- **Subculture:** A new culture system supporting a small part of cell population from an earlier culture.
- Easier to carry out, even with a simpler set-up of the laboratory.

Continuous cultures

- Also called **mass cultures**

- Cells grown in a single vessel but the nutrient medium keeps being renewed
- Two subtypes:
  - *Open type*: Cells harvested at the time of renewing the culture media
  - *Closed type*: No cell harvesting occurs. The cells coming out during the draining of the old culture medium are returned to the culture.
- Offers advantages like –
  - Ease of maintaining sterility over a long period of time
  - Less detrimental effects on mechanical failures
  - A degree of automation
  - More versatile

Requirements

1. Explant – As described earlier
2. Culture medium – Essentially same as the one required for callus growth but to achieve cell dispersal:
   - Agar is omitted
   - Auxins / Cytokinins ratio is manipulated

   The following medium was used by Reynolds and Murashige in 1979, for Tobacco suspension culture.

Constituents		Amount (in mg / litre)
1.	**Inorganic nutrients**	Same as in MS Medium
2.	**Thiamine HCl**	10
3.	**Pyridoxine HCl**	10
4.	**Nicotinic acid**	5
5.	**Myoinositol**	100
6.	**Caesin Hydrolysate**	1000
7.	**2-4 D**	2
8.	**Kinetin**	0.1
9.	**Sucrose**	30,000
10.	***p*H**	5.7

3. An arrangement for regular agitation of the culture to achieve cell dispersal

Operation

1. A mass of cells is collected – either by:
   a. Mechanical method, applying maceration of tissues

b. Enzymatic method, applying pectinase enzyme

2. Sterilization of the material – with substances like Sodium hypochlorite
3. The material is then suspended in a liquid callus induction media containing all the required nutrients and elements to allow for optimal growth which acts to turn all cells into undifferentiated cells.
4. The cell suspension is then placed on a shaker to allow the cell aggregates to disperse to form smaller clumps and single cells that are equally distributed throughout the liquid media.
5. The cells will continuously grow until one of the factors becomes limiting causing cell growth to slow.
6. Depending on culture type – it can either be maintained as a Batch Culture or Continuous Culture.

Applications

An overview of applications in brief

Plant cell culture can be used for:

1. Investigate the properties and potentialities of plant cells like control cell division, control of cell to cell interaction, totipotent abilities of the cells etc.
2. Isolate some natural chemical product of interest such as Single cell proteins, secondary metabolites etc. Some examples of important secondary metabolites are:
    - Pyrethrin from *Chrysanthemum*
    - Quinine from *Cinchona officinalis*
    - Vincristine from *Catharanthus roseus*
    - Atropine from *Atropa belladona*
3. Bring about **biotransformation** (the chemical modification made by an organism on a chemical compound) for example:
    - Conversion of Digitoxin in Digoxin by *Digitalis* cells
    - Conversion of Hydroquinone into Arbutin by *Datura* cells
    - Conversion of Steviol into Stevioside by *Stevia rebaudiana* and *Digitalis purpurea*
4. Select the desirable mutants or cell lines in crop improvement programmes, because free cells in a culture permit quick administration and withdrawal of chemical substances in order to identify the mutants or cell lines modified in a desired way.

Certain applications in detail

1. Mutant selection

An important use of cell cultures is in mutant selection in relation to crop improvement. The frequency of mutations can be increased several fold through mutagenic treatments, and millions of

cells can be obtained. If selection of mutations will be exercised as cellular level, no chimeras will be obtained, which is sometimes a drawback of mutation breeding methods, where mutants are selected at the level of whole plants. A large number of reports are now available where mutants have been selected at the cellular level. The cells are often selected directly by adding the toxic substance against which resistance is sought in the mutant cells. Using this strategy, cell lines resistant to amino acid analogues, antibiotics, herbicides, fungal toxins (causing diseases), etc. have actually been isolated. Cell cultures of *Nicotiana* were selected, for resistance against several herbicides, which included the following: amitrol, sodium chlorate, chlorsul-furon and sulformeturon methyl.

2. Production of secondary metabolites

It is well known that plants are an important source for a variety of chemicals used for a variety of purposes including pharmacy, medicine and industry. In recent years, plant cell suspension cultures and immobilized cells are being utilized for the production of these chemicals on a commercial scale, due to the following advantages over extraction from whole plants: (i) the yield and quality of the product is more consistent in cell cultures because it is not influenced by the environment, (ii) The production schedule can be predicted and controlled in the laboratory or industry.

Table: Plant species and secondary metabolites obtained from them using tissue culture techniques

Plant species	Product	Activity
Catharanthus roseus	Vincristine,	Antileulkaemic
	Ajmalthine (serpentine),	Antiarthythmic
	Ajmalicine	Tranquilizer
Chrysanthemum cinerartaefolium	Pyrethrin	Insecticide (for grain storage)
Cinchona officinalis	Quinine	Antimalarial
Digitalis lanata	Digoxin, Reserpine	Cardiac tonic, Hypotensive
Dioscorea deltoids	Diosgenin, Ant fertile	
Jasminum sp.	Jasmine	Perfume
Papaver somniferum	Morphine	Analgesic, sedative
P. bracteatum	Codeine	Analgesic
Thaumatococcus daninelli	Thaumatin	Sweetener
Datura stramonium	Scopolamine	Antihypertension
Atropa belladonna	Atropine	Blocking of cholinergic
Coptis japonica	Berberine	Antibacterial
Campatotheca accuminata	Camptothecin	Anticancer
Nicotiana Tabacum	Nicotine glutathione, ubiquinone -10	Ganglion blocker, Cardiovascular agent
Coleus blumei	Rosamarinic acid	Spice, antioxidant
Morinda citrifolia (also Cassia tora)	Anthraquinones	Laxatives, dyes
Cephalotaxus harringtonia	Cephalotaxine	Antitumour
Lithospermun eryhthrorhizon	Shikonines	Dye, pharmaceutical

Stevia rebaudiana	Stevioside	Sweetner

The most important chemicals produced using cell cultures are secondary metabolites, which are defined as 'those cell constituents which are not essential for survival '. These secondary metabolites include alkaloids, glycosides (steroids and phenolics), terpenoids and a variety of flavours, perfumes, agro chemicals, etc. The yield of these chemicals in cell culture is though generally lower than in whole plants, it can be substantially increased by manipulating physiological and biochemical conditions.

3. Biotransformation

Plant cell cultures are also being utilized for producing valuable products including secondary metabolites through biotransformation, a technique also utilized with the help of microbes. In this technique, low cost precursors are used as a substrate and are transformed into value added high cost products. At least in some cases, this transformation is achieved more easily by cultured plant cells, rather than either by micro-organisms or by chemicals synthesis. Following are some examples of the use of cell cultures in biotransformation: (i) Suspension culture of *Digitalis lanata* can convert **'digitoxin'** or **'methyldigitoxin'** into medically important **'digoxin'** or **'methyl-digoxin'**, which is used for treatment of heart diseases. (ii) *Datura* cell cultures possess ability to convert **'hydroquinone'** into **'arbutin'** (used as diuretic and urinary antiseptic through glycosylation. (iii) Cell cultures of *Stevia rebaudiana* and *Digitalis purpurea*, can convert steviol into **'steviobiocide'** and **'stevioside'** which are 100 times sweeter than cane sugar.

Limitations of cell culture

Contamination is one of the largest problems when dealing with cell cultures. Contamination becomes a problem because micro-organisms can grow much faster that plant cells and take up all the nutrients preventing the plants from growing. However, several approaches are available to deal with this problem.

Plant Tissue Culture

An overview of plant tissue culture

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and Nepenthes.
- To clean particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (**totipotency**). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

Why do plant tissue culture?

- A single explant can be multiplied into several thousand plants in less than a year - this allows fast commercial propagation of new cultivars
- Taking an explant does not usually destroy the mother plant, so rare and endangered plants can be cloned safely
- Once established, a plant tissue culture line can give a continuous supply of young plants throughout the year

- In plants prone to virus diseases, virus free explants (new meristem tissue is usually virus free) can be cultivated to provide virus free plants
- Plant 'tissue banks' can be frozen, then regenerated through tissue culture
- Plant cultures in approved media are easier to export than are soil-grown plants, as they are pathogen free and take up little space (most current plant export is now done in this manner)

Overview of the plant tissue culture technique

Modern plant tissue culture is performed under aseptic conditions. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting material (explants) in chemical solutions (usually alcohol and sodium or calcium hypochlorite) is required.

Explants are then usually placed on the surface of a solid culture medium. But, they will be placed directly into a liquid medium, when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar.

A typical culture medium developed by Murashige and Skoog (also called MS medium) is as follows.

Essential element	Concentration in stock solution (mg/l)	Concentration in medium (mg/l)
Macroelements[b]		
NH_4NO_3	33 000	1 650
KNO_3	38 000	1 900
$CaCl_2.2H_2O$	8 800	440
$MgSO_4.7H_2O$	7 400	370
KH_2PO_4	3 400	170
Microelements		
KI	166	0.83
H_3BO_3	1 240	6.2
$MnSO_4.4H_2O$	4 460	22.3
$ZnSO_4.7H_2O$	1 720	8.6
$Na_2MoO_4.2H_2O$	50	0.25
$CuSO_4.5H_2O$	5	0.025
$CoCl_2.6H_2O$	5	0.025
Iron source[c]		
$FeSO_4.7H_2O$	5 560	27.8
$Na_2EDTA.2H_2O$	7 460	37.3
Organic supplement[c]		
Myoinositol	20 000	100
Nicotinic acid	100	0.5
Pyridoxine·HCl	100	0.5
Thiamine·HCl	100	0.5
Glycine	400	2
Carbon source[d]		
Sucrose	Added as solid	30 000

[a] Many other commonly used plant culture media (such as Gamborg's B5 and Schenk and Hildebrandt (SH) medium) are similar in composition to MS medium and can be thought of as 'high-salt' media. MS is an extremely widely used medium and forms the basis for many other media formulations.
[b] 50 ml of stock solution used per litre of medium.
[c] 5 ml of stock solution used per litre of medium.
[d] Added as solid.

To the nutrient medium, plant hormones are also added. They have an important effect on the rates of division and the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots. A balance of both auxin and cytokinin will often produce an unorganised growth of cells, or callus. As cultures grow, pieces are typically sliced off and transferred to new media (subcultured) to allow for growth or to alter the morphology of the culture.

As shoots emerge from a culture, they may be sliced off and rooted with auxin to produce plantlets which, when mature, can be transferred to potting soil for further growth in the greenhouse as normal plants.

Plant tissue culture applications

Plant tissue culture is used in the following main categories of applications.

- Micropropagation
- Germplasm preservation
- Somaclonal variation & mutation selection
- Embryo Culture
- Haploid & Dihaploid Production
- In vitro hybridization – Protoplast Fusion
- Industrial Products from Cell Cultures

Specific applications in the plant sciences, forestry, and in horticulture are enlisted below.

1. The commercial production of plants used as potting, landscape, and florist subjects, which uses meristem and shoot culture to produce large numbers of identical individuals.
2. To conserve rare or endangered plant species.
3. A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance.
4. Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals.
5. To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
6. To rapidly study the molecular basis for physiological, biochemical, and reproductive mechanisms in plants, for example in vitro selection for stress tolerant plants, and in vitro flowering studies.

7. To cross-pollinate distantly related species and then tissue culture the resulting embryo which would otherwise normally die (Embryo Rescue).
8. For chromosome doubling and induction of polyploidy, for example doubled haploids, tetraploids, and other forms of polyploids. This is usually achieved by application of antimitotic agents such as colchicine or oryzalin.
9. As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.
10. Certain techniques such as meristem tip culture can be used to produce clean plant material from virused stock, such as potatoes and many species of soft fruit.
11. Production of identical sterile hybrid species can be obtained.

Important types of plant tissue and organ culture

Culture types

Plant tissue cultures are generally initiated from sterile pieces of a whole plant. These pieces are termed 'explants', and may consist of pieces of organs, such as leaves or roots, or may be specific cell types, such as pollen or endosperm. Many features of the explant are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective.

Several different culture types can be developed using the principles and methods of plant tissue culture. They are examined briefly below.

Callus

Explants, when cultured on the appropriate medium, usually with both an auxin and a cytokinin, can give rise to an unorganised, growing and dividing mass of cells. It is thought that any plant tissue can be used as an explant, if the correct conditions are found. In culture, this proliferation can be maintained more or less indefinitely, provided that the callus is subcultured on to fresh medium periodically. During callus formation there is some degree of dedifferentiation (i.e. the changes that occur during development and specialisation are, to some extent, reversed), both in morphology (callus is usually composed of unspecialised parenchyma cells) and metabolism. One major consequence of this dedifferentiation is that most plant cultures lose the ability to photosynthesise. This necessitates the addition of other components—such as vitamins and, most importantly, a carbon source—to the culture medium, in addition to the usual mineral nutrients.

Callus culture is often performed in the dark (the lack of photosynthetic capability being no drawback) as light can encourage differentiation of the callus.

During long-term culture, the culture may lose the requirement for auxin and/or cytokinin. This process, known as 'habituation', is common in callus cultures from some plant species (such as sugar beet).

Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from

which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies.

Root cultures

Root cultures can be established in vitro from explants of the root tip of either primary or lateral roots and can be cultured on fairly simple media. The growth of roots in vitro is potentially unlimited, as roots are indeterminate organs. Although the establishment of root cultures was one of the first achievements of modern plant tissue culture, they are not widely used in plant transformation studies.

Shoot tip and meristem culture

The tips of shoots (which contain the shoot apical meristem) can be cultured in vitro, producing clumps of shoots from either axillary or adventitious buds. This method can be used for clonal propagation.

Shoot meristem cultures are potential alternatives to the more commonly used methods for cereal regeneration as they are less genotype-dependent and more efficient (seedlings can be used as donor material).

Embryo culture

Embryos can be used as explants to generate callus cultures or somatic embryos. Both immature and mature embryos can be used as explants. Immature, embryo-derived embryogenic callus is the most popular method of monocot plant regeneration.

Microspore culture

Haploid tissue can be cultured in vitro by using pollen or anthers as an explant. Pollen contains the male gametophyte, which is termed the 'microspore'. Both callus and embryos can be produced from pollen. Two main approaches can be taken to produce in vitro cultures from haploid tissue.

1. The first method depends on using the anther as the explant. Anthers (somatic tissue that surrounds and contains the pollen) can be cultured on solid medium (agar should not be used to solidify the medium as it contains inhibitory substances). Pollen-derived embryos are subsequently produced via dehiscence of the mature anthers.
2. Anthers can also be cultured in liquid medium, and pollen released from the anthers can be induced to form embryos, although the efficiency of plant regeneration is often very low.

Immature pollen can also be extracted from developing anthers and cultured directly, although this is a very time-consuming process.

In microspore culture, the condition of the donor plant is of critical importance, as is the timing of isolation. Pretreatments, such as a cold treatment, are often found to increase the efficiency. These pretreatments can be applied before culture, or, in some species, after placing the anthers in culture. Regeneration from microspore explants can be obtained by direct embryogenesis, or via a callus stage and subsequent embryogenesis.

Somatic embryogenesis

In somatic (asexual) embryogenesis, embryo-like structures, which can develop into whole plants in a way analogous to zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly.

- In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Though common from some tissues (usually reproductive tissues such as the nucellus, styles or pollen), direct somatic embryogenesis is generally rare in comparison with indirect somatic embryogenesis.
- In indirect somatic embryogenesis, callus is first produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus.

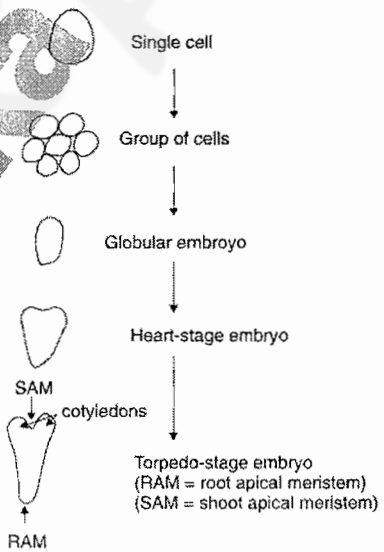

Figure: An outline of Somatic Embryogenesis

Protoplast culture and Somatic Hybridisation

The protoplast, also known as naked plant cell refers to all the components of a plant cell excluding the cell wall. Hanstein introduced the term protoplast in 1880 to designate the living matter enclosed by plant cell membrane.

The isolated protoplast is unusual because the outer plasma membrane is fully exposed and is the only barrier between the external environment and the interior of living cell.

The isolation of protoplasts from plant cells was first achieved by microsurgery on plasmolyzed cells by mechanical method (Klercker 1892). However, the yields were extremely low and this method is not useful. Cocking (1960) first used enzymes to release protoplasts. He isolated the enzyme cellulase from the culture of fungus *Myrothecium verrucaria* to degrade the cell walls.

Protoplast isolation

Protoplasts are isolated by:

1. Mechanical methods
2. Enzymatic methods

Mechanical method

In this method, large and highly vacuolated cells of storage tissues such as onion bulb scales, radish root and beet root tissue could be used for isolation. The cells are plasmolysed in an iso-osmotic solution resulting in the withdrawal of contents in the center of cell. Subsequently, the tissue is dissected and deplasmolyzed to release the protoplasts.

However, this method is generally not followed because of certain disadvantages; (i) It is restricted to certain tissues which have large vacuolated cells. (ii) Yield of protoplasts is generally very low. Protoplasts from less vacuolated and highly meristematic cells do not show good yield. (iii) The method is tedious and laborious. (iv) Viability of protoplasts is low because of the presence of substances released by damaged cells.

The mechanical method is useful when there are side effects of cell wall degrading enzymes.

Enzymatic method

Cocking in 1960 demonstrated the possibility of enzymatic isolation of protoplasts from higher plants. He used concentrated solution of *cellualse* to degrade the cell walls. However, there was little work for the next 10 years until the commercial enzyme preparations became available.

Protoplasts are now routinely isolated by treating tissues with a mixture of cell wall degrading enzymes in solution, which contain osmotic stabilizers. The relative ease with which protoplast isolation can be achieved depends upon a variety of factors which are:

- Physiological state of tissue and cell material
- Enzymes
- Osmotic conditions

The enzymatic isolation of protoplasts can be performed in two different ways.

1. **Two-step or sequential method:** The tissue is first treated with a macerozyme or pectinase enzyme which separates the cells by degrading the middle lamella. These free cells are then treated with cellulase which releases the protoplasts. In general, the cells are exposed to different enzymes for shorter periods.
2. **One step or simultaneous method:** The tissue is subjected to a mixture of enzymes in a one step reaction which includes both macerozyme and cellulase. This one step method is generally used because it is less labor-intensive.

During the enzyme treatment, the protoplasts obtained need to be stabilized because the mechanical barrier of cell wall which offered support has been broken. For this reason an osmoticum is added which prevents the protoplasts from bursting.

Protoplast purification

The enzyme digested mixture obtained at this stage would contain sub-cellular debris, undigested cells, broken protoplasts and healthy protoplasts. This mixture is purified by a combination of filtration, centrifugation and washing.

Assessing protoplast viability and density

The most frequently used staining methods for assessing protoplast viability are:

1. Fluorescein diacetate (FDA) staining method: FDA, a dye that accumulates inside the plasmalemma of viable protoplasts can be detected by fluorescence microscopy.
2. Phenosafranine staining: Phenosafranine is also used at a concentration of 0.01% but it is specific for dead protoplasts that turn red. Viable cells remain unstained by phenosafranine.
3. Calcofluor White (CFW) staining: Calcofluor White can ascertain the viability of protoplasts by detecting the onset of cell wall formation. CFW binds to the beta-linked glucosides in the newly synthesized cell wall which is observed as a ring of flurorscence around the plasma membrane.
4. Exclusion of Evans blue dye by intact membranes.
5. Observations on cyclosis or protoplast streaming as a measure of active metabolism.
6. Variation of protoplast size with osmotic changes.
7. Oxygen uptake measured by an oxygen electrode which indicates respiratory metabolism.
8. Photosynthetic studies.

However, the true test of protoplast viability is the ability of protoplasts to undergo continued mitotic divisions and regenerate plants.

Protoplast Culture techniques

Isolated protoplasts are usually cultured in either liquid or semisolid agar media plates. Protoplasts are sometimes allowed to regenerate cell wall in liquid culture before they are transferred to agar media.

Agar culture

Agar, of different qualities is available but agarose is most frequently used to solidify the culture media. The concentration agar should be chosen to give a soft agar gel when mixed with the protoplast suspension. The petridishes are sealed with paraffin and incubated upside down. By agar culture method, protoplasts remain in a fixed position so that protoplasts give rise to cell clones and allow accurate determination of plating efficiency. Once immobilized, however, hand manipulations are required for transfer to other culture media.

Liquid culture

It has been generally preferred in earlier stages of culture because (i) it allows easy dilution and transfer; (ii) protoplasts of some species do not divide in agarified media; (iii) osomotic pressure of the medium can be effectively reduced (iv) density of cells can be reduced after few days of culture. But it has the disadvantage of not permitting isolation of single colonies derived from one parent cell.

Various modifications to these culture methods have been developed. Some of which are:

- Liquid droplet method
- Hanging deoplet method
- Feeder layer
- Co-culturing

Culture medium

Protoplasts have nutritional requirements similar to those of cultured plant cells. The numeral salt compositions established for plant cell cultures have been modified to meet particular requirements by protoplasts.

Protoplast culture medium should be devoid of ammonium as it is detrimental to its survival and quantity of iron and zinc should be reduced. On the other hand calcium concentration should be increased 2-4 times over the concentration normally used for cell cultures. Increased calcium concentration may be important for membrane stability.

Mannitol and sorbitol are the most frequently used compounds for maintaining the osmolarity.

Glucose is perhaps the preferred and most reliable carbon source. Plant cells grow about equally well on a combination of glucose and sucrose, but sucrose alone may not always be satisfactory for plant protoplasts.

The vitamins used for protoplast culture include those present in standard tissue culture media. If protoplasts are to be cultured at very low density in defined media there maybe a requirement for additional vitamins. Protoplast media frequently contain one or more amino acids.

The majority of protoplast culture media contain one or more auxins plus one or two cytokinins to stimulate protoplast division and growth.

Environmental factors

Generally high light intensity inhibits protoplast growth when applied from the beginning of culture. It is better to initiate protoplast culturing in darkness or dim light for few days and later transfer the cultures to a light of about 2000-5000 lux. There are reports of better protoplast growth when the cultures are kept in continuous darkness. In contrast, it has been shown for legume species that light is necessary for initiating protoplast division. Protoplast cultures are generally cultured at

temperatures ranging between 20-28°C. A pH in the range of 5.5 to 5.9 is recommended for protoplast culture media and it seems to be satisfactory.

Protoplast development

Cell wall formation

Protoplasts in culture generally start to regenerate a cell wall within a few hours after isolation and may take two to several days to complete the process under suitable conditions. The protoplasts lose their characteristic spherical shape, which is an induction of new wall regeneration.

Growth, division and plant regeneration

Regeneration of a cell wall is not necessarily a prerequisite for the initiation of nuclear division in protoplast cultures but cell wall formation is required before cytokinesis occurs. Once a cell wall is formed, the reconstituted cells show a considerable increase in cell size. The first cell division generally occurs within 2-7 days. Protoplasts from actively dividing cell suspension enter the first division faster than those from highly differentiated cells of the leaf. The second division occurs within a week, and by the end of the second week in culture, small cell colonies are visible.

Once small colonies have formed, they should be transferred to a mannitol or sorbitol-free medium. Macroscopic colonies are transferred to an osomotic free medium to develop callus. The callus may then be induced to undergo organogenic or embryogenesis differentiation leading to the formation plants.

The first report of plant regeneration was in *Nicotiana tabacum* (Takabe et al., 1971). Since then the list of species exhibiting the totipotency has steadily increased. Several reports have suggested that frequency of plant regeneration reported from calluses derived from plant organs differ from the calluses raised from protoplasts. The calluses from intact plant organs often carry preformed buds or organized structures, while such structures are absent in the callus from protoplast origin.

Protoplast fusion and somatic hybridization

Sexual hybridization between closely related species has been used for year to improve cultivated plants. Unfortunately, sexual hybridization is limited in most cases to cultivars within a species or at best to a few wild species closely related to a cultivated crop. Species barriers thereby limit the usefulness of sexual hybridization for crop improvement.

Somatic cell fusion leading to the fomation of viable cell hybrids has been suggested as a method to overcome the species barriers to sexual hybridzation. Plant protoplasts offer exciting possibilities in the fields of somatic cell genetics and crop improvement.

The technique of hybrid production through the fusion of isolated somatic (body) protoplasts under in vitro conditions and subsequent development of their product (heterokaryon) to a hybrid plant is known as **somatic hybridization**.

This procedure eliminates sex altogether in hybridization. In somatic hybridization the nucleus and cytoplasm of both parents are fused in the hybrid cells. Sometime, nuclear genome of only one parent but cytoplasmic genes (plastome) from both the parents are present in the fused hybrid, which is known as cybrid or cytoplasmic hybrid. Thus, protoplast fusion technique can be used to overcome the barriers of incompatibility and acts as a method for the genetic manipulation of plant cells. It provides us with an opportunity to construct hybrids between taxonomically distant plant species beyond the limit of sexual cross ability. It also creates cells with new genetic, nuclear as well as cytoplamic constitutions that otherwise cannot be contained.

Techniques

Various techniques have been discovered to induce fusion to take place. Two of the most successful techniques are:

1. The addition of polyethylene glycol **(PEG method)** in the presence of a high concentration of calcium ions and a pH between 8-10
2. The application of short pulses of direct electrical current (**electro-fusion method**).

By mixing protoplasts from plants of two different species or genera, fusions may be accomplished:

- between protoplasts of the same plant where fusion of the nuclei of two cells would give rise to a homokaryon (**synkaryon**);
- between protoplasts of the same plant species (**intravarietal or intraspecific fusion**);
- between protoplasts of different plant species or genera (**interspecific or intergeneric fusion**).

Fusions of types (b) and (c) above can result in the formation of genetic hybrids (**heterokaryocytes**), which formally could only be obtained rarely through sexual crossings.

By separating the fused hybrid cells, it has been possible to regenerate new **somatic hybrid** (as opposed to sexually hybrid) plants. Some novel interspecific and intergeneric hybrid plants have been obtained by this means.

Applications

Some applications of somatic hybridization technique are tabulated below.

Somatic hybrids		Traits (resistance)
Nicotiana tabacum	+ *N. nesophila*	Tobacco mosaic virus
Solanum tuberosum	+ *S. chacoense*	Potato virus X
N. tabacum	+ *N. nesophila*	Tobacco horn worm
S. tuberosum	+ *S. brevidens*	Potato leaf roll virus Late blight and PLRV Potato virus Y
S. circalifolium	+ *S. tuberosum*	*Phytophthora*
S. melongena	+ *S. sanitwongsei*	*Pseudomonas solanacearum*
S. tuberosum	+ *S. commersonii*	Frost tolerance
B. napus	+ *B. nigra*	*Phoma lingam*
B. oleracea	+ *Sinapis alba*	*Alternaria brassiceae*
B. napus	+ *Sinapis alba*	*Alternaria brassiceae*
Citrus sinensis	+ *Poncirus trifoliata*	*Phytophthora*
Lycopersicon esculentum	+ *L. peruvianum*	TMV, spotted wilt virus, cold tolerance
S. lycopersicoides	+ *L. esculentum*	Cold tolerance
Solanum ochranthum	+ *L. esculentum*	Tomato diseases and insect pests
Brassica oleracea	+ *B. napus*	Black rot (*Xanthomonas campestris*)
B. oleracea sp. *capitata*	+ *B. oleracea* (Ogura CMS line)	Cold tolerance
S. tuberosum	+ *S. cammersonii*	Frost tolerance
R. sativa (Japanese radish)	+ *B. oleracea* var. *botrytis*	Club rot disease
B. oleracea var. *botrytis*	+ *S. alba* + *B. carinata*	*Alternaria brassicola* and *Phoma lingam*
Citrullus lanatus	+ *Cucumis melo*	Club rot resistance
Hordeum vulgare	+ *Daucus carota*	Frost and salt tolerance
Solanum melongena	+ *S. sisymbrifolium*	Nematode
S. tuberosum	+ *S. bulbocastanum*	Nematode
S. tuberosum	+ *S. bulbocastanum*	Root knot nematode
Raphanus sativus	+ *Brassica napus*	Beet cyst nematode
Sinapis alba	+ *R. sativus* + *B. napus*	Beet cyst nematode
Quality characters		
N. rustica	+ *N. tabacum*	High nicotine content
B. napus	+ *Eruca sativa*	Low erucic acid

Cybrids

Introduction to cybrids

A cytoplasmic hybrid (or **cybrid**) is a eukaryotic cell line produced by the fusion of a whole cell with a cytoplast. Thus, a cybrid can be defined as a somatic hybrid in which nucleus is derived from from one parent and cytoplasm is derived from both the parents.

Such cybrid plants can be useful in plant breeding programmes for the transfer of cytoplasmic genes.

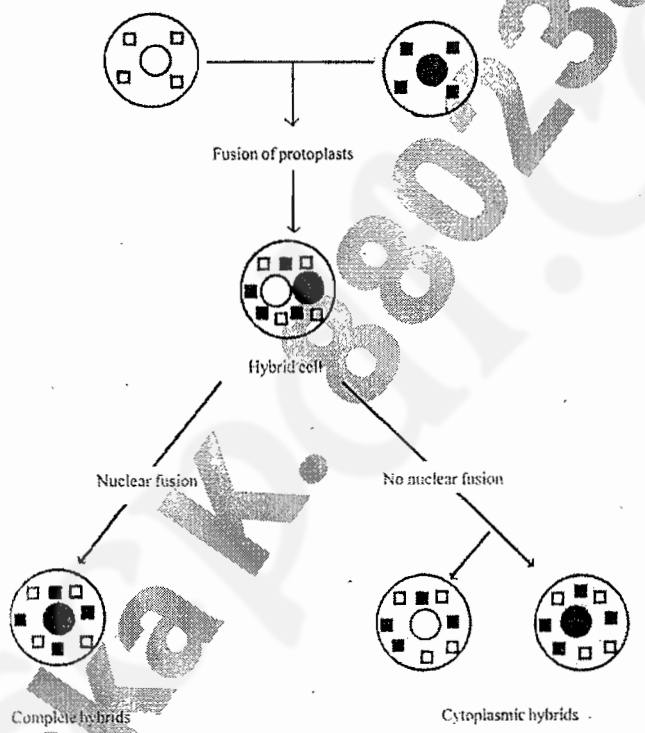

Figure: Concepts of complete hybrids and cybrids — Schematic illustration of somatic hybridization which can produce complete hybrids (left) or cytoplasmic hybrids (right).

Production of cybrids

Sexual hybridization is a precise mixture of parental nuclear genes but the cytoplasm is largely derived from the maternal parent only. In somatic hybrids, however, the cytoplasm is derived from both the parents.

By experimental methods, such somatic hybrids can also be obtained where nucleus is derived from only one parent and cytoplasm is derived from both, thus producing cytoplasmic hybrids, also called as cybrids.

Early segregation of nuclei in a fused product can be stimulated and directed so that one protoplast contributes the cytoplasm while the other contributes the nucleus alone or both nucleus and cytoplasm. There are different ways of inactivating the nucleus of one protoplast. Thus, there will be fusion between protoplasts containing the full complement of nucleus, mitochondria and chloroplasts with functional cytoplasmic component of second parent. The various approaches to achieve this type of fusion are:

1. **By application of lethal dosages of X-ray or gamma-ray irradiation** to one parental protoplast population: This treatment renders the protoplasts inactivate and non-dividing but they serve as an efficient donor of cytoplasmic organelles when fused with recipient protoplasts. *Nicotiana* protoplasts can be inactivated by 5-kr dose of X-rays. Other protoplasts may require different doses.

2. **By treatment with iodoacetate to metabolically inactivate the protoplasts**: Pretreatment with iodoacetate will cause the degeneration of non-fused and autofused protoplasts while fusion of iodoacetate pretreated protoplasts with non-treated protoplasts will cause metabolic complementation and result in viable hybrids. In an experiment, iodoacetate-treated *Nicotiana plumbaginifolia* cell suspension was fused with X-irradiated *N. tabacum* mesophyll protoplasts. All regenerated cybrid plants had *N. plumbaginifolia* morphology but most of them contained *N. tabacum* chloroplasts. The iodoacetate treatment does not impair the nucleus of the treated protoplasts. Thus, the latter can complement an X-irradiated protoplast.

3. **Fusion of normal protoplasts with enucleated protoplasts**: Enucleated protoplasts can be obtained by high-speed centrifugation (20,000-40,000x g) for 45-90 minutes in an iso-osmotic density gradient with 5-50% percoll.

4. Fusion of a normal protoplast with another in which nuclear division is suppressed.

Applications of cybrids

The importance of cybrid is in the fact that it is a hybrid cell, which combines the nuclear genome from one source with the mitochondrial and plastidial genome from another or both source. Using this powerful tool, it is possible to dissociate the genetic contribution of the organellar genome from that of the nuclear genome.

Cybridization thus opens an exciting avenue to achieve alloplasmic constitution in a single step without the need to perform a series of 8-12 time-consuming backcrosses. (**Alloplasmic lines** contain nucleus of one parent genome with the other parent's cytoplasmic constituents.)

Application of cybrids would be the directed transfer of cytoplasmic male sterility or herbicide resistance from a donor to a recipient crop plant species. Transfer of cytoplasmic male sterility (CMS) from *N. tabacum* to *N. sylvestris* by protoplast fusion was first reported by Zelcer *et al.* (1978). Resistance of plants to herbicide atrazine has been transferred from *Brassica campestris* to B. *napus* via fractionated protoplast fusions. CMS has been successfully transferred in various crop species of *Oryza, Lycopersicon, Brassica, Nicotiana,* etc.

The table below summarizes some important genetic traits transferred to plant species via the cybridisation technique.

Agronomic characters (transferred via cybrid formation)		
N. tabacum	+ *N. sylvestris*	Streptomycin resistance
N. tabacum spp.		Triazine resistance
S. nigrum	+ *S. tuberosum*	Triazine resistance
B. nigra	+ *B. napus*	Hygromycin resistance
B. napus spp.		Triazine resistance
N. tabacum	+ *N. sylvestris*	CMS
B. campestris	+ *B. napus*	CMS
N. tabacum	+ *N. sylvestris*	CMS
B. napus + *B. campestris*	+ *Raphanus sativa*	CMS and Triazine resistance
Oryza spp.		CMS
L. esculentum	+ *Solanum acaule*	CMS
B. napus	+ *B. tournefortii*	CMS

Micropropagation

Micropropagation & its need

Most methods of plant transformation applied to GM crops require that a whole plant is regenerated from isolated plant cells or tissue which have been genetically transformed. This regeneration is conducted *in vitro* so that the environment and growth medium can be manipulated to ensure a high frequency ofh regeneration. The primary aim is therefore to produce, as easily and as quickly as possible, a large number of regenerable cells that are accessible to gene transfer.

Practically any plant transformation experiment relies at some point on tissue culture. The ability to regenerate plants from isolated cells or tissues *in vitro* underpins most plant transformation systems.

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods.

Micropropagation is used • to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used • to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or • to provide a sufficient number of plantlets for those plants which do not respond well to vegetative propagation.

How do we micropropagate plants?

Selecting the right culture medium

When cultured *in vitro*, all the needs, both chemical and physical, of the plant cells have to met by the culture vessel, the growth medium and the external environment (light, temperature, etc.).

Culture media used for the *in vitro* cultivation of plant cells are composed of three basic components:

1. essential elements, or mineral ions, supplied as a complex mixture of salts;
2. an organic supplement supplying vitamins and/or amino acids; and
3. a source of fixed carbon; usually supplied as the sugar sucrose.

For practical purposes, the essential elements are further divided into the following categories:

- macroelements (or macronutrients);
- microelements (or micronutrients); and

- an iron source.

Complete, plant cell culture medium is usually made by combining several different components, as outlined in table below.

Essential element	Concentration in stock solution (mg/l)	Concentration in medium (mg/l)
Macroelements[b]		
NH_4NO_3	33 000	1 650
KNO_3	38 000	1 900
$CaCl_2.2H_2O$	8 800	440
$MgSO_4.7H_2O$	7 400	370
KH_2PO_4	3 400	170
Microelements[c]		
KI	166	0.83
H_3BO_3	1 240	6.2
$MnSO_4.4H_2O$	4 460	22.3
$ZnSO_4.7H_2O$	1 720	8.6
$Na_2MoO_4.2H_2O$	50	0.25
$CuSO_4.5H_2O$	5	0.025
$CoCl_2.6H_2O$	5	0.025
Iron source[c]		
$FeSO_4.7H_2O$	5 560	27.8
$Na_2EDTA.2H_2O$	7 460	37.3
Organic supplement[c]		
Myoinositol	20 000	100
Nicotinic acid	100	0.5
Pyridoxine-HCl	100	0.5
Thiamine-HCl	100	0.5
Glycine	400	2
Carbon source[d]		
Sucrose	Added as solid	30 000

[a] Many other commonly used plant culture media (such as Gamborg's B5 and Schenk and Hildebrandt (SH) medium) are similar in composition to MS medium and can be thought of as 'high-salt' media. MS is an extremely widely used medium and forms the basis for many other media formulations.
[b] 50 ml of stock solution used per litre of medium.
[c] 5 ml of stock solution used per litre of medium.
[d] Added as solid.

Figure: MS Medium

Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells. There are five main classes of plant growth regulator used in plant cell culture, namely:

1. Auxins;
2. Cytokinins;
3. Gibberellins;
4. Abscisic acid;
5. Ethylene.

Of the above, auxins and cytokinins are most frequently used in micropropagation. However, abscisic acid plays a role in somatic embryogenesis. The rest two hormones, gibberellins and ethylene are not applied to micropropagation.

The plant growth regulators used most commonly are naturally occurring plant hormones or their synthetic analogues.

Auxins

Auxins promote both cell division and cell growth The most important naturally occurring auxin is IAA (indole-3-acetic acid), but its use in plant cell culture media is limited because it is unstable to both heat and light.

2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used synthetic auxin and is extremely effective in most circumstances.

Cytokinins

Cytokinins promote cell division. Naturally occurring cytokinins are a large group of structurally related (they are purine derivatives) compounds. Of the naturally occurring cytokinins, two have some use in plant tissue culture media. These are zeatin and 2iP (2-isopentyl adenine). Their use is not widespread as they are expensive (particularly zeatin) and relatively unstable. The synthetic analogues, kinetin and BAP (benzylaminopurine), are therefore used more frequently. Non-purine-based chemicals, such as substituted phenylureas, are also used as cytokinins in plant cell culture media.

Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and are usually used together, the ratio of the auxin to the cytokinin determining the type of culture established or regenerated (see Figure below). A high auxin to cytokinin ratio generally favours root formation, whereas a high cytokinin to auxin ratio favours shoot formation. An intermediate ratio favours callus production.

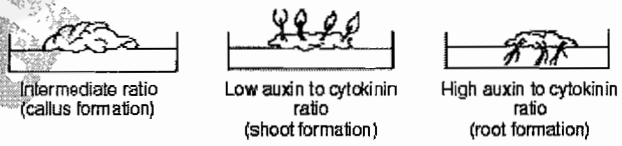

Figure: Use of auxins and cytokinins in micropropagation

The process of micropropagation

Cultures are generally initiated from sterile pieces of a whole plant. These pieces are termed 'explants', and may consist of pieces of organs, such as leaves or roots, or may be specific cell types,

such as pollen or endosperm. Many features of the explant are known to affect the efficiency of culture initiation.

Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective.

The process of micropropagation can be divided into four stages:

1. **Initiation stage**. A piece of plant tissue (called an explant) is (a) cut from the plant, (b) disinfested (removal of surface contaminants), and (c) placed on a medium. A medium typically contains mineral salts, sucrose, and a solidifying agent such as agar. The objective of this stage is to achieve an aseptic culture. An aseptic culture is one without contaminating bacteria or fungi.

2. **Multiplication stage.** A growing explant can be induced to produce vegetative shoots by including a cytokinin in the medium. As shown in the earlier figure, a cytokinin — in micropropagation — promotes shoot formation from growing plant cells.

3. **Rooting or preplant stage**. Growing shoots can be induced to produce adventitious roots by including an auxin in the medium. As shown in the earlier figure, an auxin — in micropropagation — promotes shoot formation from growing plant cells. For easily rooted plants, an auxin is usually not necessary, and many commercial labs will skip this step. "Hardening" refers to the preparation of the plants for a natural growth environment. Until this stage, the plantlets have been grown in "ideal" conditions, designed to encourage rapid growth. Due to lack of necessity, the plants are likely to be highly susceptible to disease and will be inefficient in their use of water and energy. Hardening typically involves slowly weaning the plantlets from a high-humidity, low light, warm environment to what would be considered a normal growth environment for the species in question. This stage (pretransplant) is not always performed, instead being incorporated into the last stage by encouraging root growth and hardening *ex vitro*, or in nonsterile plant media.

4. **Acclimatization**. A growing, rooted shoot can be removed from tissue culture and placed in soil. When this is done, the humidity must be gradually reduced over time because tissue-cultured plants are extremely susceptible to wilting.

Three approaches of micropropagation

There are **three major approaches** which are employed for micropropagation of different plant species.

1. Proliferation from Axillary buds
2. Induction of adventitious buds, bulbs, protocorms etc.
3. Somatic embryogenesis

Proliferation from axillary buds.

The production of plants from axillary buds or shoots has proved to be the most generally applicable and reliable method of true-to-type in vitro propagation. Two methods are commonly used:

1. Shoot culture
2. Single, or multiple, node culture.

Both depend on stimulating precocious axillary shoot growth by overcoming the dominance of shoot apical meristems.

Induction of adventitious buds, bulbs, protocorms etc.

In certain species, adventitious shoots which arise directly from the tissues of the explant (and not within previously-formed callus) can provide a reliable method for micropropagation. However, the induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived, and is highly dependent on plant genotype. In responsive plants, adventitious shoots can be formed in vitroon pieces of tissue derived from various organs (e.g. leaves, stems, flower petals or roots); in others species, they occur on only a limited range of tissues such as bulb scales, seed embryos or seedling tissues. Direct morphogenesis is observed rarely, or is unknown, in many plant genera.

Somatic embryogenesis.

Somatic embryogenesis refers to the initiation of embryos from previously differentiated somatic cells. Unlike cells of other eucaryotes, almost all plant cells have the capacity to become embryogenic under defined conditions.

In somatic (asexual) embryogenesis, embryo-like structures, which can develop into whole plants in a way analogous to zygotic embryos, are formed from somatic tissues.

These somatic embryos can be produced either directly or indirectly.

- In **direct somatic embryogenesis**, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Though common from some tissues (usually reproductive tissues such as the nucellus, styles or pollen), direct somatic embryogenesis is generally rare in comparison with indirect somatic embryogenesis. It is well studied in *Medicago*.

- In **indirect somatic embryogenesis**, callus is first produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus. Somatic embryogenesis from carrot (*Daucus*) is the classical example of indirect somatic embryogenesis.

Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage (embryo initiation), a high concentration of 2,4-D is used. In the second stage (embryo production) embryos are produced in a medium with no or very low levels of 2,4-D. in late stages, ABA is applied to invoke embryonic dormancy.

In many systems it has been found that somatic embryogenesis is improved by supplying a source of reduced nitrogen, such as specific amino acids or casein hydrolysate.

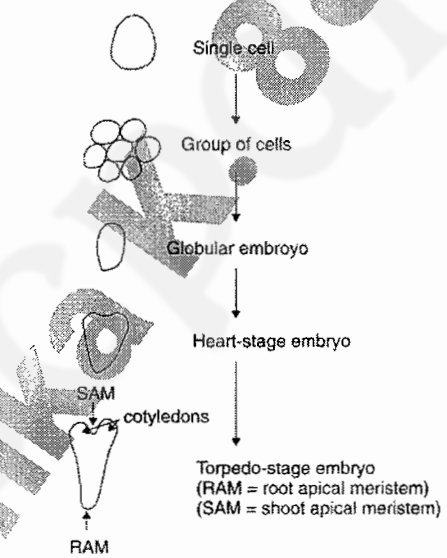

Figure: A schematic representation of the sequential stages of somatic embryo development. Somatic embryos may develop from single cells or from a small group of cells. Repeated cell divisions lead to the production of a group of cells that develop into an organised structure known as a 'globular-stage embryo'. Further development results in heart- and torpedostage embryos, from which plants can be regenerated. Zygotic embryos undergo a fundamentally similar development through the globular (which is formed after the 16- cell stage), heart and torpedo stages. Polarity is established early in embryo development. Signs of tissue differentiation become apparent at the globular stage and apical meristems are apparent in heart-stage embryos.

The somatic embryo thus formed can be preserved as a synthetic seed for a later use.

Benefits and Limitations

Micropropagation has a number of advantages over traditional plant propagation techniques:

1. Micropropagation produces clonal plants — hence preserves the genotype (for special ornamental value or some productive value).
2. Micropropagation from meristematic tissues produces disease-free plants.
3. Micropropagation produces rooted plantlets ready for growth, rather than seeds or cuttings.
4. It has an extraordinarily high fecundity, producing thousands of propagules in the same time it would take a conventional technique to produce tens or hundreds.
5. It is the only viable method of regenerating genetically modified cells or cells after protoplast fusion.
6. It is a good way of multiplying plants which produce seeds in uneconomical amounts.
7. It is a good way of multiplying plants which produce unviable seeds as in orchids.
8. Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods.
9. Micropropagation is used to provide a sufficient number of plantlets for those plants which do not respond well to vegetative propagation.
10. Micropropagation is used for conservation purposes of certain forest trees.
11. Micropropagation is widely used for large tree transgenesis.

The limitations of micropropagation include:

1. It is *very* expensive. Most plants will naturally produce seeds, which are normally disease free and will readily grow under good conditions. The number of seeds varies, but is normally acceptable for multiplication and is free. For this reason, many plant breeders will never resort to micropropagation because of the prohibitive cost.
2. It can have a labour cost of more than 70%
3. An infected plant sample can produce infected progeny. This is uncommon, as stocks are usually carefully vetted to prevent this

Mechanisation of the process would eliminate most of the labour cost associated, but this has proven difficult so far despite active attempts to develop this technology.

Somaclonal variations

Introduction to somaclonal variations

Somaclonal variations are spontaneous changes in the properties of plants or plant cells being cultured in vitro. Somaclonal variation is not restricted to, but is particularly common in plants regenerated from callus. The variations can be genotypic or phenotypic, which in the later case can be either genetic or epigenetic in origin.

In current scientific literature, somaclonal variation involves all forms of variation encountered in tissue culture. As described below, there are many reasons for spontaneous somaclonal variation. The causes can include point mutations, loss of genes, and chromosomal rearrangement. They may express themselves by altering traits controlled by many different genes (polygenic). However, the amount of variation that can be expected in vitro will vary with the clone, age of the clone, use of mutagenic agents, and use of selection pressure applied to single cells for stress conditions such as salt level, herbicides, microorganisms or their byproducts, and specific metabolites.

Somaclonal variation is a phenomenon of significant interest as it can be used for plant breeding.

The major likely benefit of somaclonal variation is in plant improvement and arieties developed by somaclonal variation exist for tomatoes, potatoes, sugar cane and others.

Some good examples of somaclonal variations in important crop plants include the following:

1. *Helminthosporium* resistance in sugar cane
2. *Phytophthora infestans* resistance in potato
3. *Pseudomonas* resistance in tobacco
4. *Fusarium oxysporum* resistance in tomato
5. *Xanthomonas oryze* resistance in rice

At a scientific level, somaclonal variation leads to the creation of additional genetic variability. Characteristics for which somaclonal mutants can be enriched during in vitro culture include resistance to disease pathotoxins, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites. Yet, it is noteworthy that positive effects of somoclonal variation are often accompanied by undesirable mutations.

Reasons behind somaclonal variations

The origins of somaclonal variation have been studied extensively, but remain largely theoretical or unknown. Some types of somaclonal variation have been transient (epigenetic), whereas others have

been either stable through repeated generations of asexual propagation or have proved to be genetically based and inherited in a Mendelian fashion.

However, from what is currently known to the scientists – the following reasons can be cited to be behind somaclonal variations.

1. Pre-existing genetic variation in the explant tissue.
2. Variants may be produced due to subtle changes because of gene mutations in cultures. Somaclonal variants for single recessive gene mutations are reported for maize, rice and wheat. Several gene mutations are reported in wheat somaclones.
3. Somatic crossing over followed by segregation may be responsible for homozygosity which in turn causes phenotypic expression of the recessive nature.
4. Numerical and structural changes in chromosomes during in vitro growth. Polyploidy, euploidy, inversion, deletion as well as translocation are reported to cause variation in regenerated plants.
5. Somatic crossing over involving symmetric and asymmetric recombination. Tissue culture may enhance the frequency of somatic crossing over.
6. Changes in DNA, isoenzyme and protein profiles have been correlated with somaclonal variations in plants including potato, rice, barley and maize.
7. Transposable elements have been reported to induce changes in strains of alfalfa and wheat.
8. Intracellular mutagenic agents produced during in vitro growth. Alteration of a single base pair results in changes of a single amino acid in polypetide sequence, thus producing somaclonal variants.
9. Variation in DNA methylation could be responsible for tissue culture-induced mutagenesis. The DNA methylation and base sequence changes are frequent in maize callus and in regenerated plants.
10. Changes in organelle DNA. The classical example of such variation in tissue culture is reported in cytoplasmically controlled male sterility. A single mutation to male fertility and toxin insensitivity was due to frame shift mutation in mitochondrial DNA.
11. Epigenetic variation. Cultured cells upon exposure to stress factor result in altered expression of traits, and these changes are temporary and are not reflected in offsprings. Such epigenetic changes in tissue culture could be due to DNA amplification, DNA methylation or transposable elements.
12. Phillips et al. (1994) suggested that somaclonal variation occurs by a stress-response mechanism.
13. Somaclonal variation can also potentially occur when plants are being regenerated from selected, successfully genetically transformed cells.

Isolation of somaclonal variants

It is grouped in two broad categories:

1. Screening, and
2. Cell selection

1. **Screening** involves the observation of large number of cells or regenerated plants for detecting variants; this is the only visible technique for isolation of mutants for yield and yield traits. This procedure is applied for isolating cell clones, which produce high amount of certain biochemicals.

2. For **selection,** a selection pressure is the basis of cell selection that allows survival or growth of desired variants only. e.g. selection of cells resistant to toxins, herbicides, high salt concentration etc. When the selection medium allows only the mutant cells to survive, it is termed as positive selection, whereas, in case of negative selection wild type cells divide and are killed by a counter selection agent like arsenate. The basis for selection of auxotroph mutants is the negative selection. Generally, two types of selection are applied:

    a. single step selection; and

    b. multi-step selection.

In the former one, the inhibitor is added to culture medium three times in excess compared to maximum inhibitory concentration (MIC) and cultures are maintained for several sub-culture regimes with the inhibitor. In a multi-step selection, a sub-lethal concentration is added to culture medium. In the subsequent sub-cultures, a gradual increase in inhibitor level is maintained. This method has been employed in tobacco for herbicide tolerance, and in maize for amino acid analogue resistance.

Applications of somaclonal variations

While recombinant DNA techniques offer promise for modification of crops, the relative paucity of knowledge of plant genetics and biochemistry has delayed development of recombinant DNA–based products using higher plants. However, somaclonal variation offers an opportunity to uncover the natural variability in plants and to use this genetic variability for new product development. Five principal advantages accruing from somaconal variations are:

1. Cost effectiveness.
2. Source of genetic variability and especially helpful in crops with a narrow genetic base.
3. Successfully removes defects in otherwise well adapted cultivars.

4. Improves various vegetative and seed propagated species.
5. Produces new variants.

Some current applications of somaclonal variations are listed below.

1. Increased genetic variability for agronomic traits: Several useful somaclonal variants showing resistance to diseases, insects and tolerance to herbicides have been isolated – as listed below.

   a. Wheat: Tolerance to drought stress; Resistance to barley yellow dwarf virus
   b. Rice: Improved lysine content
   c. *Brassica*: Salt tolerance
   d. Tobacco: Herbicide resistance
   e. Potato: Resistance to *Fusarium* sp.
   f. Sugarcane: Resistance to Eyespot
   g. *Phytophthora infestans* resistance in potato
   h. *Pseudomonas* resistance in tobacco
   i. *Fusarium oxysporum* resistance in tomato
   j. *Xanthomonas oryze* resistance in rice

2. *In vitro* selection: *In vitro* selection has been used to select agronomically desirable somaclones, including tolerance to pathotoxins, herbicides and diseases. Some examples include:

   a. Tobacco varients resistant to *Pseudomonas syringae* and *Alternaria alternata* have been obtained following selecting protoplast-derived callus on medium having pathotoxins.
   b. Somaclonal variants with increased resistance to *Fusarium oxysporum* in celery have been obtained.
   c. Herbicide resistant variants have been obtained in cell culture).

3. 'Elite' Germplasm and Commercial cultivars: Variants with improved traits have been obtained/developed giving rise to new useful germplasm, as well as cultivars

4. Improved ornamental plants: Somaclonal variation is known to be employed commercially in ornamental plants (Duncan, 1997). A wide range of somaclonal variation for flower size, plant morphology, plant height in *Begoniax elatior* plants regenerated from callus have been reported. Flower variation is reported in tissue culture - derived plants of carnation, *Chrysanthemum* and *Gerbera.*

Drawbacks of somaclonal variations

1. Instability of the variants
2. Poor plant regeneration.
3. Some somaclones possess undesirable features like sterility, etc.
4. Variation is usually not new.
5. Unpredictability associated with nature of somaclonal variation. Somaclonal variation has proved to be a serious problem for investigators and propagators who require extreme uniformity.

Embryo rescue method

Introduction to embryo culture and rescue

Embryo culture is the sterile isolation and growth of an immature or mature embryo *in vitro*, with the goal of obtaining a viable plant.

***History of embryo culture*:** The first attempt to grow the embryos of angiosperms was made by Hannig in 1904, who obtained viable plants from *in vitro* isolated embryos of two crucifers *Cochleria* and *Raphanus* (Hannig, 1904). In 1924, Dietrich grew embryos of different plant species and established that mature embryos grew normally but those excised from immature seeds failed to achieve the organization of a mature embryo (Dietrich, 1924). They grew directly into seedlings, skipping the stages of normal embryogenesis and without the completion of dormancy period. Laibach (1925, 1929) demonstrated the practical application of this technique by isolating and growing the embryos of interspecific cross *Linum perenne* and *L. austriacum* that aborted *in vivo*. This led Laibach to suggest that in all crosses where viable seeds are not formed, it may be appropriate to excise their embryos and grow them in an artificial nutrient medium. This approach is called **embryo rescue**.

Embryo culture is now a well-established branch of plant tissue culture.

There are two types of embryo culture:

1. Mature embryo culture
2. Immature embryo culture (also known as **Embryo Rescue**)

Mature embryo culture is the culture of mature embryos derived from ripe seeds. This type of culture is done when embryos do not survive *in vivo* or become dormant for long periods or is done to eliminate the inhibition of seed germination. Seed dormancy of many species is due to chemical inhibitors or mechanical resistance present in the structures covering the embryo, rather than dormancy of the embryonic tissue. Excision of embryos from the testa and culturing them in the nutrient media may bypass such seed dormancy. Some species produce sterile seeds, which may be due to incomplete embryo development. Embryo culture procedures may yield viable seedlings. Embryos excised from the developing seed at or near the mature stage are autotrophic and grow on a simple inorganic medium with a supplemental energy source.

Immature embryo culture or **embryo rescue** is described below in the next section.

Embryo rescue & its methods

Principles of embryo rescue

Application of embryo culture techniques to obtain interspecific and intergeneric hybrid plants prior to abortion of the embryos due to post fertilization hybridization barriers is known as **Embryo Rescue.** Thus, immature embryo culture or embryo rescue is the culture of immature embryos to rescue the embryos of wide crosses. This is mainly used to avoid embryo abortion with the purpose of producing a viable plant. Wide hybridization, where individuals from two different species of the same genus or different genera are crossed, often leads to failure.

There are several barriers, which operate at pre- and post-fertilization levels to prevent the successful gene transfer from wild into cultivated species. The pre-fertilization barriers include all factors that hinder effective fertilization, which is usually due to inhibition of pollen tube growth by the stigma or upper style. Post-fertilization barriers hinder or retard the development of the zygote after fertilization and normal development of the seed. This frequently results from the failure of the hybrid endosperm to develop properly, leading to starvation and abortion of the hybrid embryo or results from embryo-endosperm incompatibility where the endosperm produces toxins that kill the embryo.

Raghavan (1976) has discussed evidence, which suggests that embryos of inviable hybrids possess the potential for initiating development, but are inhibited from reaching adult size with normal differentiation. Endosperm development precedes and supports embryo development nutritionally, and endosperm failure has been implicated in numerous cases of embryo abortion. Endosperm failure generally results in abnormal embryo development and eventual starvation. Thus, isolation and culture of hybrid embryos prior to abortion may circumvent these strong post-zygotic barriers to interspecific and intergeneric hybridization. The production of interspecific and intergeneric hybrids is the most conspicuous and impressive application of embryo rescue and culture technique, particularly for subsequent valuable gene transfer from wild species.

Methods of embryo rescue

The underlying principle of embryo rescue technique is the aseptic isolation of embryo and its transfer to a suitable medium for development under optimum culture conditions. With embryo culture, there are normally no problems with disinfecting. Florets are removed at the proper time and either florets or ovaries are sterilized. Ovules can then be removed from the ovaries. The tissue within the ovule, in which the embryo is embedded, is already sterile. The embryos can then be aseptically removed from

the ovules. Utilization of embryo culture to overcome seed dormancy requires a different procedure. Seeds that have hard coats are sterilized and soaked in water for few hours to few days. Sterile seeds are then split and the embryos excised.

Embryo culture, in which embryos are excised and placed directly onto culture media is the most widely used embryo rescue procedure. For all embryo rescue procedures, the probability of success increases with the maturity of embryos.

The most important aspect of embryo culture work is the selection of medium necessary to sustain continued growth of the embryo. The type of nutrient medium needed for rescuing embryos is strongly dependent on the stage of embryo development. Young embryos require a complex medium with high sucrose concentration, while more mature embryos can usually develop on a simple medium with low level of sucrose.

In most cases, a standard basal plant growth medium with major salts and trace elements may be utilized. Mature embryos can be grown in a basal salt medium with a carbon energy source such as sucrose. However, young embryos in addition require different vitamins, amino acids, and growth regulators and in some cases, natural endosperm extracts. Young embryos should be transferred to a medium with high sucrose concentration (8-12%) which approximate the high osmotic potential of the intracellular environment of the young embryo-sac, and a combination of hormones which supports the growth of heart-stage embryos (a moderate level of auxin and a low level of cytokinin). Reduced organic nitrogen as aspargine, glutamine or casein hydrolysate is always beneficial for embryo culture. Malic acid is often added to the embryo culture medium. After one or two weeks when embryo ceases to grow it must be transferred to a second medium with a normal sucrose concentration, low level of auxin and a moderate level of cytokinin which allows for renewed embryo growth with direct shoot germination in many cases. In some cases where embryo does not show shoot formation directly, it can be transferred to a medium for callus induction followed by shoot induction. After the embryos have grown into plantlets *in vitro,* they are generally transferred to sterile soil and grown to maturity.

Applications of embryo rescue

In plant breeding programmes, not all desirable crosses are successful. When the fertilization is normal and the hybrid embryo capable of normal development aborts due to inadequate supply of nutrition from endosperm or due to endosperm-embryo incompatibility it is possible to raise complete hybrid plants through rescuing the embryo. This method has been profitably used in the hybrid seed production.

1. The cross between *Lilium speciosum-album* and *L. auratum* produced seeds with large amount of endosperm and long embryo. When these seeds were sown the embryo degenerated and no seedlings were formed because of embryo-endosperm incompatibility. However, when the immature embryos were rescued and grown on nutrient medium, a full hybrid plant was produced.
2. The caryopses formed in crosses between *Hordeum vulgare* and *H. bulbosum* are frequently devoid of endosperm. Because of this, embryos die prematurely. By excising the embryo and culturing it 14-28 days after pollination, seedling was raised.
3. A cross between diploid *Brassica chinensis* and tetraploid *B. pekinensis* produced triploid hybrid embryo which failed to mature because of early endosperm degeneration. By culturing hybrid embryo on a medium containing diffusate of young seeds of *Lupinus luteus*, Inomata (1967) was able to raise complete hybrid plants.
4. Through embryo rescue techniques, hybrids have been raised from several interspecific crosses of *Oryza,* which are otherwise impossible to obtain.
5. Crossing cultivated tomato *(Lycopersicon esculentum)* with wild tomato *(L. peruvianum)* has been considered desirable from the point of view of transferring disease resistance from the latter to the former. This cross fails because of embryo abortion and no viable seeds are formed (Thomas & Pratt, 1981). By the rescuing of poorly developed hybrid embryo, diploid or tetraploid hybrid plants were obtained.
6. In cotton the culture of hybrid embryo has given encouraging results for obtaining viable seedlings in crosses between *Gossypium arboreum* (2n = 26) and G. *hirsutum* (4n = 52).
7. *In vitro* zygotic embryo culture continues as a means of rescuing hybrid embryos during interspecific gene transfer. Parker and Michalis (1986) employed this technology to recover hybrids of *Phaseolus vulgaris* and *P acutifolius. P acutifolius* has improved tolerance to common bean bacterial blight (*Xanthomonas campestris*) than *p. vulgaris* (common field bean). Embryos from crosses between these species frequently abort. Embryo rescue assisted the transfer of improved bacterial blight tolerance to economically important field beans.
8. Embryo rescue technique has been utilized in the transfer of cytoplasmically inherited herbicide tolerance from *Brassica napus* and *B. oleracea.*
9. Orchids are difficult to propagate since their seeds lack any stored food and the embryo is virtually naked. In many orchids, embryo development is incomplete at the time the seed matures. The Singapore Botanic Gardens have recently succeeded in producing 1,431 hybrids of orchids through embryo rescue technique. Callus induction from embryos of orchids can be very useful in obtaining more hybrids.
10. More recently, a number of hybrids have been successfully raised through embryo rescue technique in many cases like *Hordeum vulgare* x *Secale cereale, H. vulgare* x *Agropyron repens,* and *H. vulgare* x *Triticum sp.*

Anther culture and pollen haploids

Background

The purpose of anther and pollen culture is the production of haploid plants by the induction of embryogenesis from repeated divisions of microspores or immature pollen grains. The chromosome complement of these haploids can be doubled by colchicine treatment or other techniques to yield fertile homozygous diploids. The resultant diploids can be used in plant breeding to improve crop plants.

The haploid nature of embryoids should be determined by standard chromosome staining procedures (acetocarmine or Feulgen reaction) prior to colchicine treatment. Similarly, the effect of colchicine on chromosome doubling needs to be monitored by chromosome staining.

Materials

- Tobacco buds
- Sharp pointed forceps, surgical scissors
- 95% ethanol
- Petri dishes of culture medium (1/2 strength ms, 2% sucrose 0.8% agar, glutamine [800 mg/liter], serine [100 mg/liter])

Methods

1. Obtain two buds at the appropriate stage. This occurs in tobacco when the sepals and the petals in the bud are the same length.
2. Holding the bud by the pedicel between the thumb and first finger, dip the entire bud in 95% ethanol for 15 seconds
3. Remove bud and allow excess alcohol to drip off.
4. With a pair of sterile forceps, remove the outer layer of tissue, the sepals.
5. Next, remove the inner layer of tissue, the petals, exposing the anthers.
6. Open the petri dish containing the medium for the induction of haploids. Remove each anther from the bud and drop it onto the medium. Do not damage the anther or include any filament tissue.
7. Repeat for another bud.

8. When finished, seal the plates and place in incubator (25°C).
9. In 2–3 weeks examine for somatic embryo initiation. Embryoid-forming cells are characterized by dense cytoplasmic contents, large starch grains and a relatively large nucleus. Embryoids appear opaque among translucent cells. Embryoids also exhibit high dehydrogenase activity and can be detected by tetrazolium staining.

Details

Haploid tissue can be cultured in vitro by using pollen or anthers as an explant. Pollen contains the male gametophyte, which is termed the 'microspore'. Both callus and embryos can be produced from pollen. Two main approaches can be taken to produce in vitro cultures from haploid tissue.

The first method depends on using the anther as the explant. Anthers (somatic tissue that surrounds and contains the pollen) can be cultured on solid medium (agar should not be used to solidify the medium as it contains inhibitory substances). Pollen-derived embryos are subsequently produced via dehiscence of the mature anthers. The dehiscence of the anther depends both on its isolation at the correct stage and on the correct culture conditions. In some species, the reliance on natural dehiscence can be circumvented by cutting the wall of the anther, although this does, of course, take a considerable amount of time. Anthers can also be cultured in liquid medium, and pollen released from the anthers can be induced to form embryos, although the efficiency of plant regeneration is often very low. Immature pollen can also be extracted from developing anthers and cultured directly, although this is a very time-consuming process.

Both methods have advantages and disadvantages. Some beneficial effects to the culture are observed when anthers are used as the explant material. There is, however, the danger that some of the embryos produced from anther culture will originate from the somatic anther tissue rather than the haploid microspore cells. If isolated pollen is used there is no danger of mixed embryo formation, but the efficiency is low and the process is time-consuming.

In microspore culture, the condition of the donor plant is of critical importance, as is the timing of isolation. Pretreatments, such as a cold treatment, are often found to increase the efficiency. These pretreatments can be applied before culture, or, in some species, after placing the anthers in culture.

Plant species can be divided into two groups, depending on whether they require the addition of plant growth regulators to the medium for pollen/anther culture; those that do also often require organic supplements, e.g. amino acids. Many of the cereals (rice, wheat, barley and maize) require medium supplemented with plant growth regulators for pollen/anther culture.

Regeneration from microspore explants can be obtained by direct embryo-genesis, or via a callus stage and subsequent embryogenesis.

Haploid tissue cultures can also be initiated from the female gametophyte (the ovule). In some cases, this is a more efficient method than using pollen or anthers.

The ploidy of the plants obtained from haploid cultures may not be haploid. This can be a consequence of chromosome doubling during the culture period. Chromosome doubling (which often has to be induced by treatment with chemicals such as colchicine) may be an advantage, as in many cases haploid plants are not the desired outcome of regeneration from haploid tissues. Such plants are often referred to as 'di-haploids', because they contain two copies of the same haploid genome.

Applications of pollen haploids

1. *Development of pure homozygous lines*: In the breeding context, haploids are most useful as source of homozygous lines. The main advantage is the reduction in time to develop new varieties. A conventional plant-breeding program takes about 6-8 years to develop a pure homozygous line, whereas by the use of anther/microspore culture, the period can be reduced to few months or a year. Thus, homozygosity is achieved in the quickest possible way making genetic and breeding research much easier. Homozygosity is still more important for those plants, which have a very long juvenile phase (period from seed to flowering) such as fruit trees, bulbous plants and forestry trees. Even if repeated self-pollination is possible, achievement of homozygosity in this group of plants is an extremely long process.

2. *Hybrid development*: As a result of complete homozygosity obtained from diploidization of haploids, one can rapidly fix traits in the homozygous condition. Pure homozygous lines can be used for the production of pure F_1 hybrids.

3. *Induction of mutations*: Haploid cell cultures are useful material for studying somatic cell genetics, especially for mutation and cell modification. Majority of mutations induced are recessive and therefore they are not expressed in the diploid cells because of the presence of dominant allele. Single cells and isolated pollen have the advantage over the entire plant in that they can be plated and screened in large numbers, in a manner similar to microbiological technique. Mutants, which are resistant to antibiotics, herbicides, toxins, etc., have been isolated in a number of plant species. By subjecting haploid *Nicotiana tabacum* cells to methionine sulfoximine, Carlson (1973) regenerated mutant plants, which showed a considerably lower level of infection to *Pseudomonas tabaci.*

4. *Induction of genetic variability*: By anther culture not only haploids but also plants of various ploidy levels and mutants are obtained and can be incorporated into the breeding programs.

5. *Generation of exclusively male plants*: By haploid induction followed by chromosome doubling it is possible to obtain exclusively male plants. For example, in *Asparagus officinalis* male plants have a higher productivity and yield earlier in the season than female plants. If haploids are produced from anthers of male *Asparagus* plants (XY) these are either X or Y, chromosome doubling of Y results in super male plants YY which can subsequently be vegetatively propagated.

6. *Cytogenetic research*: Haploids have been used in the production of aneuploids. Monosomics in wheat, trisomies with 2n = 25 in potato, and in tobacco nullisomics were derived from haploids.

7. *Significance in the early release of varieties*: Based on anther culture many varieties have been released. In Japan, a tobacco variety F-211 resistant to bacterial wilt has been obtained through anther culture. In *Brassica napus,* anther derived doubled haploid lines had low erucic acid and glucosinolate content. Similarly, in sugarcane, selection among anther culture derived haploids led to the development of superior lines with tall stem and higher sugar content.

8. *Hybrid sorting in haploid breeding*: One of the essential steps in haploid breeding involves selection of superior plants among haploids derived from F_1 hybrids through anther culture. It is properly described as hybrid sorting and virtually means selections of recombinant superior gametes. The haploid method of breeding involving hybrid sorting is considered superior to pedigree and bulk methods, firstly, because the frequency of superior gametes is higher than the frequency of corresponding superior plants in F_2 generation, and secondly, because haploid breeding reduces significantly the time required for the development of a new variety.

9. *Disease resistance*: Haploid production has been used for the introduction of disease resistance genes into cultivars. An established cultivar is crossed with a donor for disease resistance. Either F_1 or F_2 anthers are plated and haploids are developed. These haploids are screened for resistance and then diploidized. Resistance to barley yellow mosaic virus has been introduced into susceptible breeding lines by haploid breeding. Rice varieties have been developed with blast resistance genes, high yield and good quality using haploids integrated in conventional breeding approaches.

10. *Insect resistance*: A medium-late maturing rice variety, *Hwacheongbyeo* derived from anther culture showed resistance to brown plant hopper. This variety was also resistant to blast, bacterial blight and rice tenui virus, and showed cold tolerance.

11. *Salt tolerance*: Salt tolerant breeding lines have been developed in different crop species, which have been integrated in conventional breeding. Miah *et al.* (1996) developed doubled haploid salt tolerant rice breeding line that showed greatly elevated salt tolerance.

12. *Doubled haploids in genome mapping*: A rather recent application of DH lines is their use in genome mapping. For molecular screening studies, a much smaller sample of doubled haploids is required for desirable recombinants. In a population of DH lines, the identification of markers is much more secure, as most intermediate phenotypic expressions are excluded due to heterozygosity. A gene will segregate in a 1:1 ratio for both molecular marker and the phenotype at the plant level. DH is used for genome mapping for major genes and/or quantitative traits in barley, rice, oilseed rape, etc.